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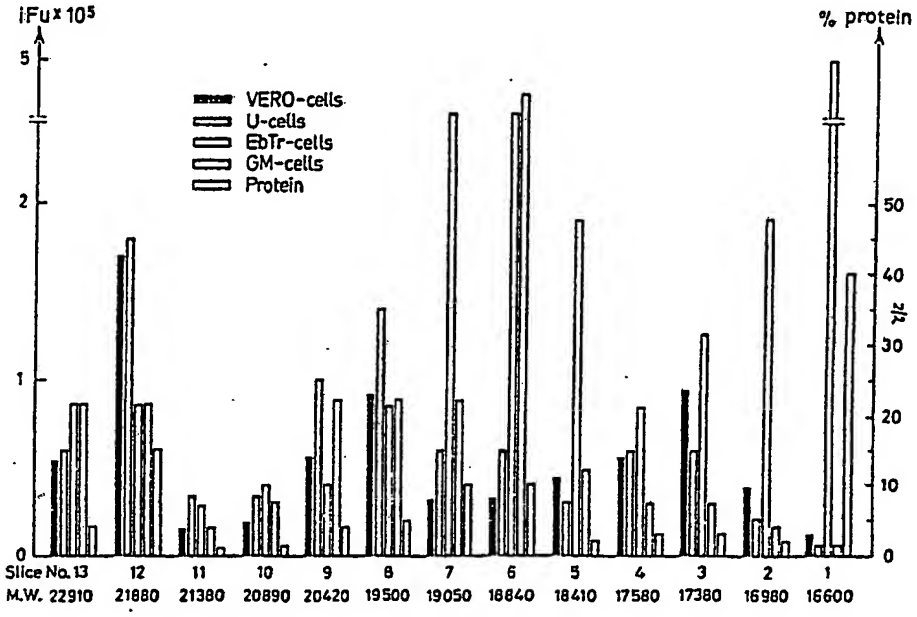


(57) Abstract

HuIFN-α proteins which under SDS PAGE conditions adapted for separation show 13 sharp stained protein bands at 16,600, 16,980, 17,380, 17,580, 18,410, 18,840, 19,050, 19,500, 20,420, 20,890, 21,380, 21,880, and 22,910 'Daltons', each of the proteins having antiviral interferon activity in various interferon test systems as illustrated in Fig. 2. Rabbit antibodies raised against each of the 12 individual species from 16,980 to 22,910 'Daltons' will react against all of these species. The interferon proteins are produced by subjecting a solution containing human Le form interferon proteins to antibody affinity chromatography.

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# SUBJECTS RELATING TO HUMAN INTEFERON-α SUBTYPE PROTEINS AND CORRESPONDING ANTIBODIES

International Patent Application No. PCT/DK80/00024 discloses pure human Le form interferon proteins, antibodies directed against them, and methods for obtaining pure human Le form interferon proteins. (According to the International Interferon Nomenclature Committee, the recommended designation for human Le form interferon proteins is now HuIFN-α proteins, and this designation will be used in the following specification and claims.)

As used herein, the term "protein" includes "glycoprotein".

In International Patent Application No. PCT/DK80/00024, the pure HuIFN-α proteins were characterized by their behaviour in a defined SDS PAGE system, where they appeared - after loading around $1x10^6$ units of HuIFN-α to one single slot - as six stained protein bands having antiviral interferon activity, that is strong bands at 18,410 Daltons and 20,180 Daltons, respectively, a medium-strong band at 20,420 Daltons and just visible bands at 19,500 Daltons, 21,130 Daltons, and 23,440 Daltons, respectively (said Dalton molecular weights being subject to an experimental accuracy of ±200 Daltons), the peaks of antiviral interferon activity coinciding exactly with the stained protein bands, said SDS PAGE acrylamide gradient showing essentially no other stained protein regions.

International Patent Application No. PCT/DK80/00024 also discloses the obtainment of antibodies by immunization against the individual stained SDS PAGE HuIFN-α bands.

In the further investigations of the individual pure HuIFN-α proteins, employing one of the very simple and convenient purification sequences disclosed in International Patent Application No. PCT/DK80/00024, that is, using a combination of gel filtration

and antibody affinity chromatography using antibodies raised against the pure HuIFN-α proteins, it has now been found that the pure interferon protein preparation, when subjected to SDS PAGE investigation in a system adapted especially for separation, yields a band pattern (comprising essentially only interferon proteins) which is even more detailed than the pattern disclosed in International Patent Application No. PCT/DK80/00024.

The more detailed pattern comprises 13 individual stained protein bands having antiviral activity (although to a varying degree) in the human system (using e.g. VERO-cells or U-cells). It was furthermore found that the same 13 species also had varying degrees of activity in the bovine system (using EbTr-cells). Of special interest is the fact that one of the individual species (at 16,600 "Daltons") obtained when using antibody raised against an eluate containing all of the species described in International Patent Application No. PCT/DK80/00024, or rasied against the species at about 18,410 Daltons, shows a remarkably high activity in the bovine system (using EbTr-cells) and an almost negligible activity in the human system (that is, a fraction of this species yielding >500,000 units in the bovine system yielded only a few thousand units in the human system - so little that it is even conceivable that this amount derives from an unprecise slicing of the gel fractions (experiments in progress)). On the whole, it has been found that there is a general trend towards increased bovine activity among the various species when the molecular weight decreases from about 20,000 Daltons. (In this connection it is noted that while the SDS PAGE system defined in International Patent Application No. PCT/DK80/00024 is a gradient gel which is acknowledged as being suitable for molecular weight determinations, the SDS PAGE system used for the new development constituted by the present invention is a system which is adapted to separation, and the "molecular weights" assessed in the new system may therefore vary to some degree from the molecular weights of the same species in the previous system, for which reason Dalton molecular weight designations determined in the new system will be stated in citation marks as "Daltons".) The new investigation described

herein of the interferon proteins purified as described above by gel filtration and antibody affinity chromatography using antibodies rasied against the 18,410 Daltons species or raised against an eluate containing all of the proteins disclosed in International Patent Application No. PCT/DK80/00024 showed that the original 18,410 Daltons species comprised at least 3 - 4 components which could be separated in the new SDS PAGE system (which is described under "Materials and Methods" herein).

One most remarkable fact is that about 40% of the total interferon proteins found in the purified interferon preparation is located in the above-mentioned 16,600 "Daltons" species which shows a very high antiviral activity in the bovine system, but almost negligible activity in the human system. It is also very interesting that antibodies directed against the 20,420 Daltons and the 23,440 Daltons species described in International Patent Application No. PCT/DK80/00024, and, quite generally, antibodies raised against any of the species above 18,410 Daltons, do not neutralize this bovine species (or neutralize this species to an extremely low extent). This means that in antibody affinity chromatography using antibodies directed against any of the species above 16,600 "Daltons", the bovine species will not be found in the eluates. Thus, one of the aspects of the present invention is that it makes it possible to remove bovine species from the other species having activity both in the human and in the bovine system.

In the SDS PAGE system used for the separation according to the present invention, at a total interferon load of 4 - 5 millions IFU, the pure HuIFN-α proteins show 13 stained protein bands, all having antiviral interferon activity, the bands appearing at

16,600, 16,980, 17,380, 17,580, 18,410, 18,840, 19,050, 19,500, 20,420, 20,890, 21,380, 21,880 and 22,910.

"Daltons", (the "Dalton" molecular weights being subject to an system (which is described under "Materials and Methods" herein). showing essentially no other stained protein regions.

The position of the individual HuIFN-α proteins appear from Fig. 1, and the antiviral interferon activity determined for each of the proteins in various interferon test systems appears from Fig. 2.

With reference to Fig. 1, the relative intensities of all the 13 bands were recorded. All 13 fractions were then titrated in 4 different cell systems comprising three human cell systems and one bovine cell system. The human cells were VERO-cells, U-cells, and GM-cells, and the bovine cells used were EbTr-cells. VSV (vesicular stomatitis virus) was used as challenge virus for all four cell systems. Each fragment slice (from SDS PAGE) was titrated in all four systems. The respective interferon titers appear from Fig. 2. As will be noted, all 13 fractions contain a distinct bovine interferon activity which more or less correlates to the human interferon activities measured in the various human systems. However, fraction No. 1 with an apparent molecular weight of 16,600 "Daltons" has almost no interferon activity in the human system, especially so when the human activity is compared to the amount of protein. It appears from Fig. 1 that slices Nos. 1 and 2 are located very close to each other. The absolute distance between these two slices is just about 1 mm. Thus, one could assume that the small amount of interferon activity in slice 1 possibly stems from slice No. 2 due to difficulties in obtaining absolutely "clean" slices. If one compares the bovine activity of slice 1 with the protein contents, there seems to be a fair degree of agreement between the amount of bovine interferon activity and the amount of protein. Thus, it is tempting to suggest that fraction No. 1 is composed solely of a bovine species produced (or generated by degradation) in the human leukocyte system. When a gel filtered human leukocyte interferon as previously described is purified by using an antibody column using antibodies obtained by immunizing a rabbit with the 21880 "Daltons" species in analogy with the above described 18,410

Daltons species, a similar protein and interferon profile is obtained, except that the bovine species is missing (or is present only to an extremely low extent). All the other species are apparently present. This indicates that the 16,600 bovine species does not cross-react with the other 12 human interferon species. It should also be noted that the remaining 12 species all contain a certain bovine activity together with their human activity (vide Fig. 2). Fig. 2 also indicates the above-mentioned general trend towards bovine activity with lower molecular weight, indicating that bovine activity is increased at the expense of the human interferon activity as the apparent molecular weight is decreased, cf. slices Nos. 5, 4, 3, 2, and 1.

At present, it is not known what are the implications of the presence of the bovine species in the HuIFN-α preparation. Several possibilities exist. One possibility is that the 16,600 "Daltons" species is derived from the remaining 12 species by a proteolytic degradation. However, this is not so likely since it is unlikely that all 12 species should decompose yielding one single protein as decomposition product. It is not known at present whether the bovine species has a biological function in the human interferon system. It has previously been described (Gresser, I, Baudu, M.T., Brouty-Boye, D., Tovey, M. (1974): Pronounced antiviral activity of human interferon on bovine and porcine cells, Nature 251, 543-545) that human leukocyte interferon contains bovine activity (the so-called cross activity). Other authors, including Braude, I.A., Lin, L.S., Stewart, W.E. (1981): Isolation of a biologically active fragment of HuIFN-α, Interferon Res. 1, 245-251, have also described that human leukocyte interferon contains bovine activity. One major discovery according to the present invention is that it is possible to isolate and characterize the 16,600 "Daltons" species as being an interferon species completely different from the other human species, i.e. the fact that the 16,600 "Daltons" species is immunologically distinct from the other HuIFN-α species. There are experimental indications supporting a notion that the bovine species at 16,600 "Daltons" might be important for stabilization/expression of biological activity (experiments in progress).

According to one aspect of the present invention, the fact that an antibody column against the 21,880 "Daltons" species will only recognize the 12 species having predominantly human activity, while an antibody column against the 18,410 Daltons species will recognize all 13 species, may be utilized to isolate the bovine species from the remaining 12 species, e.g. as follows: About 6 million units of crude leukocyte interferon is gel filtered, and after dialysis versus PBS, the interferon is loaded to a tandem column system comprising an anti-21,880 "Daltons" column connected with the anti-18,410 Daltons column. All the human interferon activity will be caught by the anti-21,880 column, while the bovine activity will pass the anti-21,880 column. The anti-18,410 column will catch the bovine species. The two columns are disconnected, washed and eluted separately. From the column against the 21,880 species, all the interferon species 1 to 12 are recovered. From the 18,410 column, only the bovine species with an apparent molecular weight of 16,600 "Daltons" will be recovered.

It is not known at present whether any of the individual species of the 12 human species and the bovine species is able to potentiate the action of either HuIFN-β HuIFN-β or different forms of β or α or whether any of these species potentiate the actions or the action of HuIFN-γ.

One aspect of the invention relates to HuIFN-α proteins which under the above conditions show the above pattern of stained bands, except when identical to proteins disclosed in International Patent Application No. PCT/DK80/00024.

Another aspect of the present invention relates to each individual protein having antiviral interferon activity which is one of the components having one of the molecular weights stated above.

As is known from International Patent Application No PCT/DK80/00024, human lymphoblastoid (Namalva) Le form proteins comprise, to the extent of about 85% thereof, HuIFN-α

proteins. It is also known that recombinant DNA techniques may be used to produce HuIFN-α proteins and proteins having the significant antigenic interferon-characterizing determinants thereof. The interferon preparation from which the proteins of the invention may be isolated may, therefore, not only comprise human leukocyte interferon preparations, but also any other preparation which contains proteins showing the antigenic properties of one or more of the HuIFN-α protein species of this invention.

Aspects of the invention appear from the appended claims. Quite generally, the interferon proteins of the present invention will be applicable for the same useful purposes as the interferon proteins disclosed in International Patent Application No. PCT/DK80/00024 and will be applied in the same or analogous manner. Similarly, the production and purification of the proteins of the invention may be performed in the same or analogous manner as described in International Patent Application No. PCT/DK80/00024.

Therefore, for a more detailed discussion of the application and production of the proteins of the present invention, the disclosure of International Patent Application No. PCT/DK80/00024 is incorporated herein by reference. With respect to the purification, however, it should be noted that according to the present invention, it has been found that the use of a purification stage comprising ligand chromatography is not a necessary stages in the purification, for which reason it is preferred to pass the interferon-containing fractions from the gel filtration directly to antibody affinity chromatography, omitting any intermediary ligand chromatography.

According to one special aspect of the present invention monospecific antibodies produced in various animals by means of the individual HuIFN-α species of the present invention and of International Patent Application No. PCT/DK80/00024 are very useful for establishing immunoassay systems (e.g., ELISA and RIA) based on radioactivity or enzyme activity. The antibodies can be used both for detection of antibodies to interferon itself and to interferon proteins present in partially pure interferon preparations. The

antibodies may also be useful for assays for determining interferon activity.

Antibodies derived from animals such as rabbits may be more advantageous than hybridoma antibodies for these purposes, considering that it is well known that the avidity of rabbit antibodies is considerably higher than the avidity of hybridoma-produced antibodies.

(According to another aspect of the invention, antibodies produced in animals, such as rabbits, against a specific interferon preparation (e.g. using an interferon preparation used clinically) render it possible to estimate the andibody response against the interferon proteins and against the contaminating proteins in the interferon preparation in question and may be used, e.g., as a positive control in experiments for assessing whether a patient treated with the interferon preparation develops antibodies against the preparation, vide below.)

Furthermore, rabbit antibodies raised against each of the 12 individual species of the HuIFN-α (except the bovine species) will react against all 12 human species of the HuIFN-α. In other words, all the 12 human HuIFN-α species which show predominant activity in the human system, cross-react immunologically 100%, whereas the bovine species (16,600 "Daltons") is immunologically distinct (in the rabbit system). On the other hand, rabbit antibodies raised against any combination of the interferon proteins which includes the bovine species, including the combination of the total of the interferon proteins described in International Patent Application No. PCT/DK80/00024, will react with all of the 13 interferon proteins. This gives the advantage that it is possible to design the determination technique so that it will either include or exclude the bovine species. One particularly important utilization of this aspect of the invention is the use of two types of antibody determination (that is, using antibodies raised against the pure HuIFN-α proteins on the one hand and antibodies raised against PIF on the other hand) for monitoring a patient who is receiving human interferons

(leukocyte or Namalva interferons) or any recombinant DNA-derived interferon which cross-reacts immunologically with the above-mentioned species. Such monitoring is performed according to the ELISA or RIA technique such as described below. Using this type of combined determination, it is possible to follow the patient's response to the interferon administered, vide the comments below. Thus, it would be possible, by performing the relevant analysis, to assess whether the patient is developing antibodies against the administered interferon proteins themselves (if the determinations using antibodies raised against HuIFN-α are positive), or against contaminating proteins (if the determinations using antibodies raised against HuIFN-α are negative, but the determinations using antibodies raised against PIF are positive). Alternatively, antigen preparations of a specific activity of $10^6$ - $10^9$ (which may be prepared by methods known per se, e.g. by the antibody techniques described in the present application and in International Patent Application No. PCT/DK80/00024, can also be used. The same principles may also be used for monitoring patients receiving preparations containing HuIFN-β or HuIFN-γ, and these utilizations also constitute aspects of the present invention.

For detection of antibodies against HuIFN-α, the following procedure is used, which is exemplified (with another protein as the target) in Methods of Enzymology, Vol. 70, p. 419 - 439 (Enzyme Immunoassay ELISA and EMIT by E. Engvall (eds.: Van Vunakis and Langone):

HuIFN-α (most preferably in a purified preparation, specific activity 1 - 100 x $10^6$ units/mg protein) is coated to a surface (e.g., a plastic surface, such as NUNC radioimmunoassay plates, or another suitable matrix), e.g., at 4°C. The remaining binding sites are either saturated by adding 0.1% BSA for 1 - 3 hours at 4°C, or the surface is washed in a buffer containing a mild detergent such as Tween 80 or Triton X-100 which will inhibit further bindings of proteins to the said surface. The anti-interferon sample (e.g., a serum sample from a patient receiving interferon treatment) in the above-mentioned buffer is contacted with the surface for 3 - 20

hours at, e.g., 4°C, and after washing (3x) with the said buffer, a predetermined amount of a different species of anti-IgG, radioactively labelled or enzymatically labelled (e.g., phosphatase or horseraddish peroxidase) is added to the buffer, and the mixture is further incubated 3 - 20 hours, e.g., at 4°C. If radiolabelled antibodies are used, the bound radioactivity is estimated according to standard procedures. If enzyme labelling is used, the relevant substrate is first added to the washed surface, using an appropriate substrate buffer (optimum pH for the enzyme system) including said detergent (incubation, e.g., for 1 - 12 hours at room temperature). The change in colour is measured by spectrophotometry. A high development in colour is taken as a sign that the patient has developed antibodies against the interferon in question or against the relevant impurities, or against both, (vide above). Like all immunoassays, these measurements must include proper controls to establish the background in a given system. Very often, the undesired cross-reaction between the two different antisera used is observed. This may be obviated by simply adding the relevant solution of the antigens causing the unspecific background. (For example, one may add sheep immunoglobulin-depleted serum to the rabbit anti-interferon serum, etc., or one may add immunoglobulin-depleted human serum to the rabbit anti-interferon sera, etc.)

Instead of coating the said surface with antigen (that is, purified interferons, specific activity 1 - $100 \times 10^6$ units/mg protein), a "double layer" technique may be used: first, the said surface is coated with either anti-HuIFN-α or anti-PIF (an antiserum raised against partially purified interferon, vide "MATERIALS AND METHODS" below, using about 1 µg/ml of the relevant IgG solution. (In certain occasions, the anti-serum may also be suitable). The antibody-coated surface is allowed to react with the relevant interferon preparation, and after washing, the anti-interferon-containing sample is allowed to react with the antibody-bound antigens (including interferon), and the above-described further procedure is followed.

The determinations for assessing any immunological reaction on preparations containing HuIFN-β- or HuIFN-γ-antigenic determinants are performed in exactly analogous manner.

According to a particular aspect of the present invention, the loading of the interferon-containing solution to the antibody affinity chromatography column is performed at a pH in the range of 5 - 7, as contrasted to the normal pH of 7.2. It is contemplated that at this lower pH, the binding of the interferon to the antibody affinity chromatography column is slightly looser, resulting in a higher recovery of biological interferon activity.

According to another aspect of the invention (which, if desired, can be combined with the above-mentioned loading at lowered pH), the coupling of the antibody to the matrix is performed under slightly reversible denaturing conditions, without affecting the binding capacity, e.g., in 4 - 8 M urea, in particular 4 - 6 M urea such as 4 - 5 M urea. There are indications that such reversible denaturation of the immunoglobulins results in a looser binding of the antigen in question (e.g. interferon) in the subsequent antibody affinity chromatography and, thus, in a higher recovery of the antigen (e.g., recovery of a higher interferon activity). In the present context, the term "denaturation" designates the phenomenon that the immunoglobulin molecule, which, in its native state, exists as a globular protein, becomes substantially uncoiled. In the present aspect of this invention, the denaturation is performed as a reversible denaturation, which means that the uncoiled protein is able to substantially revert to its native configuration after removal of the denaturant. Examples of useful denaturants for the reversible denaturation are urea in the stated molar range of 4 - 8, or urea derivatives, such as guanidine hydrochloride, in the range of 4 - 8 molar, or KSCN, etc. The method of reversibly denaturing immunoglobulins is known per se, but it is believed to be novel to reversibly denature an immunoglobulin during its binding to an antibody affinity chromatography column and thereafter remove the denaturant prior to using the column.

The contemplated beneficial effect of the partial or reversible denaturation is that the uncoiled or denatured immunoglobulin will bind to the matrix in a substantially uncoiled or linear configuration, which will expose several otherwise "hidden" binding sites to the matrix, thus binding the immunoglobulin more firmly to the matrix, concomitant with a somewhat looser, but still sufficiently firm, binding to the antigen in question (due to the denatured configuration determinants proper of the immunoglobulin), and a lesser amount of non-specific binding of proteins due to the uncoiled character of the immunoglobulin (whereby the hydrophobicity of the matrix is lowered).

Quite generally, it is often a major problem to elute small amounts of protein which have been specifically bound to an antibody column. If too small amounts of protein are present, absorptions or non-specific inactivations may often occur. One method for avoiding this comprises the experimental conditions below (chromatography in the presence of substances which stabilize the protein without changing the antigenic properties thereof with respect to the antibodies in question):

Human leukocyte interferon was gel-filtered according to the usual procedure in the presence of 0.1% Triton X-100. Gel-filtered material was dialysed against PBS containing 0.1% Triton X-100 and PBS including 0.1 molar sodium chloride, pH 7.2. This solution was supplied to an equilibrated antibody column which had been equilibrated with suitable buffer including 0.1% Triton X-100, and the usual antibody affinity chromatography procedure was executed in the presence of 0.1% Triton X-100. It was noted that the recovery from such an antibody affinity chromatograpy could be increased considerably compared to chromatographies in which the Triton X-100 was not included. It has also been found that SDS (for stabilization purposes) can be omitted when purifying interferon, provided that the Triton X-100 is included in the process as described above. This is considered advantageous since it has not previously been possible to purify small amounts of interferon completely by means of the antibody columns without using SDS as

a stabilizing agent in the eluate (omission of SDS caused a drastic reduction of the interferon yield (from 80% to about 10%).

MATERIALS AND METHODS.

Interferon assays were performed according to the well-known standard method (Berg K., Sequential Antibody Affinity Chromatography of Human Leukocyte Interferon, Scand. J. Immunol. 6, 77 - 86 (1977)) using VERO cells (monkey kidney cells), U-cells (human amnion cell line), GM-cells (trisomer for chromosome 21), DbTr-cells (bovine cells), and Vesicular Stomatitis Virus (VSV) as challenge virus. All interferon units (IFU) are expressed in international reference units (69/19 B units) (69/19 B reference was obtained from MRC, Mill Hill, U.K.)

Interferon. Crude human leukocyte interferon was produced according to the method as described by Cantell (Cantell, K., Hirvonen, S., Mogensen, K. E. and Pyhälä, L., Human leukocyte interferon: production, purification, stability and animal experiments. In: The Production and use of Interferon for the Treatment and Prevention of Human Virus Infections pp. 35 - 38, Waymouth, C. (ed.). Proceedings of a Tissue Culture Association Workshop held at Lake Placid, 1973 (In Vitro Monograph, volume 3), Tissue Culture Association, Rockville, Md.) using Sendai virus as interferon inducer. Partially purified interferon (PIF) with a specific activity of $5 \times 10^5$ IFU/mg protein was obtained from crude concentrated human leukocyte interferon (CIF) by ethanolic precipitation as described by Cantell, K., Hirvonen, S., Mogensen, K. E. and Pyhälä, L., loc. cit.

Interferon neutralization for determining anti-interferon was performed in a micro-assay system in the following manner: 20,000 VERO cells per well were seeded in 100 µl medium and kept at 5% $CO_2$ in a humidified cabinet. On day 2 the medium was removed from the cells, and each well received 100 ml of a dilution (in medium) of the antiserum, containing an interferon concentration of 6 - 8 IFU/ml (the serum and interferon had been preincubated at 37°C for 1 h). On day 3 the medium was removed, and all the wells received 100 µl VSV (diluted to $10^{-3.5}$ in medium). On day 4 the CPE (cytopathogenic effect) was determined, and 50%

destruction was taken as the end point for the determination of the anti-interferon titer. The titers are expressed as interferon neutralization units (IFU-NU) per ml.

Chemicals. CNBr was from Fluka (stored at -20°C). Sodium dodecylsulphate (SDS), specially pure for electrophorese, was purchased through British Drug House (BDH). Soyabean Trypsin Inhibitor (STI) and L-Lysine were obtained from Sigma. Sepharose 4B and CNBr-activated Sepharose were supplied by Pharmacia (Denmark).

Binding Procedures. The covalent binding of the immunoglobulins to Sepharose 4B was done as previously described by K. Berg in Scand. J. Immunolog., 6, 77 - 86, (1977). Only 80 - 85% of the immunoglobulins were deliberately bound. Binding in 4 - 8 M urea was also found advantageous, yielding higher recovery of interferon of the same or slightly higher purity. Thus, one example of a binding buffer for this purpose is 0.1 M sodium bicarbonate, pH 8 - 9.5, e.g. 8.5, including 0.3 M sodium chloride and 4 - 8 M urea, e.g. 4 - 6 M urea, such as 4 - 5 M urea.

Protein determinations were made by a modification of the Lowry procedure (Berg K., Sequential Antibody Affinity Chromatography of Human Leukocyte Interferon, Scand. J. Immunol., 6, 77 - 86 (1977)) which permitted detection of 1 - 2 µg/ml as the lowest level of proteins detectable (using an LKB Calculation Absorptioner Ultralab System). Crystalline bovine serum albumin was used as a standard protein. To determine the protein concentration of the purified interferon (1 - 5 µg in total) the following procedure was adopted: SDS was added to a final concentration of 0.1%. The lyophilized protein sample was further examined on an SDS-polyacrylamide gel electrophorese (SDS PAGE, see later), subsequent to a dialysis versus distilled water. The intensity of the stained protein bands was compared with known standards in different amounts (see later, under SDS PAGE), and the total amounts of proteins were estimated The deviations were 5 - 10%, with the lowest detectable level of proteins being 0.1 µg (in total). The results

from this method will serve as a rough estimate, rather than as an actual measurement.

Affinity chromatographies were performed at 4°C. The gel suspensions were degassed before packed into the columns. Packing was performed by washing with 3 - 5 bed volumes of loading buffer, using a peristaltic pump. Samples (100 µl) for interferon titrations were taken from either pools or individual fractions and titrated on the same day or frozen in plastic tubes (-20°C) and titrated later. The dilutions were made in medium (incl. 10% calf serum).

Antibody affinity chromatography was essentially done as described by Berg (Sequential Antibody Affinity Chromatography of Human Leukocyte Interferon, Scand. J. Immunol., 6, 77 - 86 (1977)). As loading buffer was used 0.1 M NaOA/0.3 M NaCl at pH 7.2 (flow rate 40 ml/h). Stepwise elution was performed with 0.1 M HOAc/0.3 M NaCl including a minute amount of citric acid (enough to keep the pH firmly at 2.4). When not operated, the column was stored at 4°C in PBS 1 M NaCl including Penicillin, Streptomycin, Gentamycin and Chloramphenicol (1% of each). Before using the column for purification purposes, it was first washed with 100 ml of loading buffer followed by 10 ml of eluting buffer and finally equilibrated with 20 - 30 ml of loading buffer. This washing-cycle was necessary to avoid "spontaneous" proteins, especially when working with interferon of specific activities above $10^7$ IFU/mg proteins. The plastic tubes used for collecting the interferon eluate were pre-wetted with 100 µl of 1% SDS.

SDS PAGE. The purified, concentrated interferon preparations were analyzed for polypeptides components on SDS PAGE slab gels using 20 cm long separating gels, 0.75 mm thick (Bio Rad model 221: Dual vertical slab gel electrophoresis cell) and 7 - 10 cm long stacking gel. The first 10 cm consisted of a 12 - 22% acrylamide gradient, and the remaining 10 cm consisted of 22% acrylamide. The preparation of the gels was performed by the method described in Knight, E., Interferon: Purification and initial characterization from human diploid cells. Proc. natn. Acad. Sci. USA 73,

520 - 523 (1976). The discontinuous buffer system, as described by Laenomli (Laenomli, U. K., Cleavage of Structural Proteins During Assembly of the Head of Bacteriophage T4, Nature 227, 680 - 685 (1970)) was used. The gel was pre-cooled for 2 h (10°C) before starting the actual electrophorese which was performed overnight (10°C) at constant effect (LKB power supply), starting out with 10 mA (and about 20 V). Samples to be analyzed were dissolved (or diluted) in 0.1 M Tris, HCl (pH 6.8) 2.5% SDS and 5% glucose including a tracking dye (sample buffer).The gel was stained in Comassie Blue (1.25 mg/ml in 50% methanol, 40% $H_2O$ and 10% acetic acid), without prior fixation, for 15 minutes at room temperature under constant rocking, and destained in 7% acetic acid (5% methanol). The gels were dried on paper of a good quality (for example, Whatman Chromatographic paper (17 mm)) under heat and vacuum using a gel dryer (Bio Rad, gel slab dryer, model 224). Solutions of five different molecular markers, from 0.1 µg to 10 µg of each marker per 20 µl, - Lactalbumin (14,400 Daltons); Soyabean Trypsin Inhibitor (20,100 Daltons); Carbonic Anhydrase (30,000 Daltons); Ovalbumin (43,000 Daltons); Bovine Serum Albumin (67,000 Daltons); Phosphorylase (94,000 Daltons) (obtained as an electrophoresis calibration kit (Pharmacia, Denmark)) - were subjected to SDS PAGE and stained. It should be noted that molecular weights assessed in this manner are subject to experimental accuracy of about ±100 "Daltons". The stained protein bands were compared with the corresponding bands obtained from a parallel SDS PAGE of a purified interferon preparation and the total concentration of interferon proteins was estimated. For obtaining a biological profile from an SDS PAGE, the part of the gel intended for interferon determination, was cut from the remainder gel and kept at 4°C (in a humidified box) on a glass plate. The main part of the gel was stained for 15 minutes; after destaining for additionally 3 - 5 minutes, weak bands were clearly seen on a blue background, whereby the precise location of the protein bands corresponding to 14,000 and 30,000 "Daltons" could be established. The unstained part of the gel was cut, so it only contained proteins between 14,000 and 30,000 "Daltons" and was further subdivided in 1 mm pieces by sharp knives. The interferon

from these slices was eluted with 0.5 ml 0.1 M SDS subsequent to a complete mincing by means of a teflon rod. After 5 h at room temperature (rocking) the interferon activity of the supernatant was determined. The individual fractions were frozen at -20°C without any additives.

EXPERIMENTAL SECTION.

Preparation of Pure HuINF-α Proteins.

Concentration of crude human leukocyte interferon. To 3 liters of crude human leucocyte interferon was added KSCN up to a concentration of 0.5 M at pH 7.2. The pH was lowered by addition of 1N HCl to 4.5 (magnetic stirring) whereby a protein precipitate containing the interferon (and part of the impurities) was obtained. The precipitate was dissolved in 150 ml of PBS (phosphate buffered saline, pH 7.2) including 1 M NaCl and 25% by volume of ethylene glycol and dialyzed thoroughly versus 3 times 2 liters of the same buffer at 4°C. The specific activity of the crude concentrated human leukocyte interferon (HuLeCIF) was 5 - 10 x $10^3$ IFU/mg protein. The recovery was about 98%.

Gel filtration. A 100 cm long column (2.6 cm in diameter, Pharmacia K 2.6/100) was packed with Ultrogel AcA 5/4 (LKB Denmark) in PBS containing 1 M NaCl and 25% by volume of ethylene glycol at 4°C (pH 7.2). After washing the column with 3 bed volumes of buffer, the column was stabilized. 10 - 15 ml of HuLeCIF (prepared as described above in 25% by volume of ethylene glycol, 1 M NaCl in PBS, pH 7.4) were loaded to the column, and the column was "eluted" with the loading buffer, the fractions being assayed for interferon activity. The interferon-containing fractions were pooled, and 85 - 95% of the original interferon activity was recovered. The specific activity of the gelfiltered human leukocyte interferon-containing eluates was close to 1,000,000 IFU/mg protein, corresponding to a purification factor of 200. As determined by means of molecular markers, the molecular weight of the interferon

corresponds to a range of 10,000 - 20,000 Daltons. Titrations of individual fraction revealed only one broad peak, with a maximum at 18,000 Daltons.

The gel filtration curve for the above-described gel filtration of HuIFN-α is shown in Fig. 5 in International Patent Application No. PCT/DK80/00024. It is clearly seen that the interferon activity is effectively separated from the major part of the proteins.

Antibody Affinity Chromatography. The gel filtered interferon preparation was dialyzed against PBS,. pH 7.2, and was thereafter loaded to an equilibrated (against loading buffer (0.3 NaCl, 20 mM PB, pH 7.2). After a thorough wash with the loading buffer (until the OD curve at 280 nm has decreased to the base line), the interferons were eluted with eluting buffer consisting of 0.2 M acetic acid including 0.3 M NaCl (including a minute amount of citric acid to keep the pH firmly at 2.4). For stabilization of the pure interferon proteins, the tubes in which the eluate from the antibody column is collected (fraction size 2 ml) have been pre-wetted with 100 µl of a 1% SDS solution each. After pooling of the interferon-containing eluate, additional SDS is added up to a total concentration of 0.1% by weight.

The pooled interferon-containing eluate stabilized with 0.1% SDS is transferred to a 20 ml stainless steel tube pre-cooled to 0°C in an ice bath. After 15 minutes, a precipitate is formed. The precipitate is isolated by centrifugation at 20,000 rpm at 4°C for 20 minutes. The supernatant is discarded (no interferon activity), and the precipitate is redissolved in 4 ml of 8 M urea and transferred to a Millipore concentration cell, size 8 ml, filter 10,000 molecular weight cut, and concentrated to about 100 µl at room temperature. Thereafter, additional 4 ml 4 M urea (p.a.) was added to the concentrate, and the solution was concentrated to about 100 µl at room temperature. Finally, 1 - 3 ml of distilled water was added, and the solution was concentrated to a volume of 20 µl and mixed with 20 µl SDS sample electrophoresis buffer. 20 µl of the resulting solution ($1\text{-}10\times10^6$ units in total) was used for characterization as described in the section "SDS PAGE" below.

SDS PAGE. The SDS PAGE electrophoresis was performed as described under "MATERIALS AND METHODS" above. The stained slab of the electrophoresis of the pure human leukocyte interferon proteins (5-7x$10^6$ units) is shown in Fig. 1. Fig. 1 also shows that 13 different species of HuIFN-α were separated and characterized. Thus the original 18,410 Daltons band as shown in Fig. 1 of International Patent Application No. PCT/DK80/00024 could be resolved further into 4 separate interferon peaks (slices 1 - 4, Fig. 1) of which the major protein (containing 40% of the total protein of all 13 species as judged by the intensity of the stains) is located at 16,600 "Daltons", slice No. 1 in Fig. 1. The interferon activity data shown in Fig. 1 were obtained in the same manner as described in International Patent Application No. PCT/DK80/00024 (using VERO cells). It is to be noted that apart from the protein bands shown in Fig. 1 which all contained interferon activity in the human (and the bovine) system, vide the discussion of Fig. 2 below, there was no other visible protein band in the SDS PAGE. It should also be noted that the biological peaks coincided exactly with the location of the proteins.

Production of Pure Interferon Proteins.

HuINF-α is purified according to the Experimental Section of International Patent Application No. PCT/DK80/00024. Thus, from 10 million units of crude HuIFN-α, about 5 x $10^6$ units were obtained in the last step which includes the addition of 0.1% SDS for stabilization purposes. After performing electrophoresis as described in International Patent Application No. PCT/DK80/00024, about 6 to 7 bands were seen subsequent to staining and destaining. The 18,410 Daltons band, the 20,180 Daltons band and the 23,440 Daltons band were excised from the gel and eluted by means of 0.01% SDS at 15°C for 2 - 8 hours subsequent to a gel-mincing done by means of a Teflon rod into 0.5 ml of 0.01% SDS buffer and were then titrated for interferon activity. As appears from Table I below, the following units were obtained in each fraction:

Table I.

| HuIFN-α Species | Interferon recovered (Mv) |
|---|---|
| 18,410 | 500,000 - 1,000,000 units |
| 20,180 | 50,000 - 200,000 units |
| 23,440 | 500,000 - 1,000,000 units. |

The total recovery of the interferon activity from such an SDS PAGE experiment usually amounted to 15 - 40% (most often around 30%). All these electrophoreses were done without any mercapto ethanol (which otherwise would destroy more than 95% of the biological activity).

(The destruction of human leukocyte interferon in the presence of SDS and mercaptoethanol has been described previously in the literature. The present inventor has confirmed it on a number of occasions. It was noticed that the protein pattern in an SDS PAGE, when mercaptoethanol was included in the buffers, yielded a protein pattern essentially identical with that seen when no mercaptoethanol was included in the electrophoresis buffers or sample buffers.)

Raising Antibodies Against HuIFN-α Proteins

The above procedure was followed bi-weekly, and the SDS PAGES were stained for 15 minutes in room temperature and destained for 10 minutes in 7% acetic acid, 5% methanol and washed for 3 minutes in distilled water, whereafter the interferon protein bands at 18,000, 20,000 and 22,000 were excised. Each gel slice was smashed with a Teflon rod and suspended in 0.5 ml 0.01% SDS and mixed with an equal volume of Freund's complete adjuvant before

being injected into rabbits. (The suspension must be able to enter the small 1 ml syringe, which is secured by filling and emptying the syringe with the same suspension several times.) The rabbits are each injected with the respective interferon-containing gel suspensions subcutaneously bi-weekly into the back. This procedure is followed bi-weekly for 5 months. After 5 months of this regimen, all three rabbits used developed antibodies as seen in Table II.

Table II

| number of immunization | 1 | 2 | 3 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|
| 18,410 | - | - | - | 20 | 1000 | 3000 | 5000 | 5000 |
| 20,180 | - | - | - | 10 | 10 | 200 | 200 | dead |
| 23,440 | - | - | - | 5 | 10 | 50 | 50 | 100 |

After the 5 months of immunisation, 50 to 60 ml of blood were taken from each rabbit (18,410 and 23,440, cf. Table II). The IgG immunoglobulins were isolated from the sera by means of the well-known technique involving the use of protein A coupled covalently to Sepharose 4B. Thus, aliquots of 10 ml of antisera were loaded to such an equilibrated column (the usual antibody affinity buffers (vide "Materials and Methods" above) were also used in this case). The size of the protein A-Sepharose column was 50 ml. After loading the 10 ml of serum to the column, the column was washed thoroughly subsequent to elution. The pooled eluate was titrated in the neutralization assay as described in "Materials and Methods" above, whereby it was found that 90% neutralizing activity was recovered. No neutralizing activity was found in the wash. After dialysis against 0.1 molar sodium bicarbonate, pH between 8 and 9, the IgG was bound covalently to CNBr activated Sepharose 4B from Pharmacia using 1 g (dry weight) of the activated Sepharose,

yielding a 2 to 3 ml antibody column, vide "Materials and Methods" above. The column (18,410) was checked by loading it with 5 million units of gel-filtered crude HuINF-α, specific activity of about $1 \times 10^6$ units per mg protein. More than 98% of the interferon activity was removed by the column; almost no interferon was found in the wash. The elution was performed by level lowering pH as usual to 2.4 (tubes prewetted with 0.1% SDS (recovery between 50% and 80%)). The pooled eluate containing the interferon was examined in the separation SDS PAGE described in "Materials and Methods", and the protein pattern as described below in connection with Fig. 1 was observed. The specific activity amounts to $10^8 - 10^9$ units per mg protein. All the proteins seen in the stained and destained gel contain interferon activity. Recovery was moderate (30-40%).

Similar experiments were performed with the 23,440 rabbit column. This antibody column was also able to bind at least $2 \times 10^6$ units of gel-filtered HuIFN-α which is a surprising finding comparing the neutralization titer (50 neutralizing units per ml) of the serum which would indicate the binding of 7 - 10 x 10 ml x 50 neutralizing units per ml = 5000 interferon units using 10 ml of the serum. Apparently, the neutralization test is not an important measure for exploring the binding ability of a given anti-interferon serum. As the purity of the immunogen is increased, the antibodies produced might be of a kind which might even not be detected at all in the usual neutralization tests (alternatively, to a very low extent). Recovery was rather moderate, 20-30%.

The eluate from the 23,440 column was concentrated and examined in a similar SDS PAGE as described above. Again, essentially the same protein pattern was observed as seen above, except for the 18,000 "Daltons" range in which a major part of the proteins seemed to be missing, corresponding to the 40% which is represented by the bovine species. It (A small column made by means of the 20,180 rabbit supported likewise the same results). Thus, it can be concluded that all human species are antigenically identical in the human system (using rabbit antibodies) which directly sup-

ports the previous findings in International Patent Application No. PCT/DK80/00024.

It was the different intensity of the protein stain noticed at the 18,000 location as seen in the SDS PAGE system used according to International Patent Application No. PCT/DK80/00024 of eluates from the 18,410 column compared to eluates from the 23,440 column that prompted a redesign of the SDS PAGE system to develop a system which would permit an even better resolution between the individual interferon species.

As appears from "Materials and Methods", the new modified SDS PAGE system consists of a 5% stacking gel which is identical with the stacking gel described in International Patent Application No. PCT/DK80/00024. Then follows a 12 - 22% gradient of polyacrylamide gel, 10 cm long, and finally a 10 cm long gel with a constant acrylamide gel concentration (22%). All the technical details and buffers and materials are spelled out in "Materials and Methods". As mentioned above, the molecular weights determined in the new SDS PAGE system are not directly related to the molecular weights determined in the SDS PAGE system used according to International Patent Application No. PCT/DK80/00024, which is due to the fact that in the new system, the gel concentration is constant over a certain range (10 cm).

Now, the following experiment was done:

10 million units of interferon was gel-filtered according to the method described in "Materials and Methods". After dialysis against PBS including 0.1 mol of sodium chloride, pH 7.2 (standard condition), the interferon was loaded to the 18,410 antibody column. The column was washed thoroughly and eluted by means of the usual eluting buffer. The 0.1% SDS-containing eluate was concentrated by the SDS precipitation procedures described in International Patent Application No. PCT/DK80/00024 and examined in the new SDS PAGE system, including staining, destaining and elution. As seen in Fig. 1, 13 individual protein bands were observed.

Each of these 13 protein bands had interferon activity. When comparing the range around 16,000 to 18,000 "Daltons" in Fig. 1 with the SDS PAGE pattern illustrated in Fig. 1 in International Patent Application No. PCT/DK80/00024, it can be seen that the 18,410 Daltons species as described in International Patent Application No. PCT/DK80/00024 has been further dissolved into 3 - 4 individual species. As mentioned above, the 16,600 "Daltons" species as seen in Fig. 1 contains roughly 40% of the total proteins present in such a purified human interferon protein preparation which consists only of pure interferon proteins. Furthermore, it will also be noted that the 16,600 "Daltons" species has surprisingly low activity in the human system. However, when titrating all the 13 species in a bovine cell system, the 16,600 "Daltons" species has a distinct and high activity. Thus, the 16,600 "Daltons" species is not just an impurity. Rather, it is an interferon protein which has almost no activity or alternatively a very low activity in the human system, but a very high activity in the bovine system.

Now, an analogous experiment was performed using the 23,440 column. 10 million units of crude human leukocyte interferon were processed in the same manner as described above. When examining the purified eluates eluted from the 23,440 antibody column in the new modified SDS PAGE system, the distinct protein at 16,600 "Daltons" was lacking completely (or was present only to an extremely low extent). When comparing the two experiments, the following can be deduced: The 16,600 "Daltons" species does not cross-react antigenically with the remaining human interferon species.

Antibodies in rabbits directed against all the human species, including the bovine species, have been produced by inoculating a rabbit with the total eluates from the last antibody affinity chromatography step as described in International Patent Application No. PCT/DK80/00024. Such an eluate comprising 1 to 2 million units of interferon stabilized with 0.1% SDS was injected bi-weekly into rabbits together with Freund's complete adjuvant subcutaneously for 3 to 4 months without any further SDS PAGE separations. Antibodies against interferon were developed after 3 to 4 months

during this regimen. The titers were around 300,000 - to 500,000 neutralizing units per ml. An antibody column was constructed according to the above procedure and loaded with gel-filtered human leukocyte interferon. The eluate was examined in SDS PAGE, and the usual 13 bands were seen. From time to time, it was noticed that two protein bands appeared around 10,000 "Daltons". At present, it is not known whether these protein bands represent some minor impurities bound non-specifically to this column or whether they represent interferon proteins with molecular weights lower than the 16,600 "Daltons" previously observed.

The recovery of this antibody affinity chromatography was distinctly increased when the coupling of the immunoglobulins were performed under mildly reversible denaturing conditions, such as 4-5M urea. Under these circumstances, recovery was consistently 80-85%, as compared to 50-100% (unconsistent) when no urea was incorporated in the coupling buffer.

Claims.

1. HuIFN-α proteins which under the SDS PAGE and staining conditions defined herein at a total interferon load of $5x10^6$ IFU show 13 sharp stained protein bands having antiviral interferon activity at

16,600, 16,980, 17,380, 17,580, 18,410, 18,840, 19,050, 19,500, 20,420, 20,890, 21,380, 21,880, and 22,910

"Daltons", (the "Dalton" molecular weights being subject to an experimental accuracy of ± 100 "Daltons"), the SDS PAGE showing essentially no other stained protein regions.

2. Each individual protein having antiviral interferon activity which is a component of the HuIFN-α interferon proteins claimed in claim 1.

3. A protein having antiviral interferon activity as claimed in claim 2 appearing at 16,600 ± 100 "Daltons".

4. A protein as claimed in claim 3 having immunological properties distinct from the remaining 12 species.

5. A protein constituting about 40% of the pure HuIFN-α proteins, said protein having only negligible activity in the human system and a very high activity in the bovine system and being immunologically distinct from the HuIFN-α proteins that have appreciable activity in the human system.

6. A protein having antiviral interferon activity as claimed in claim 2 appearing at 16,980 ± 100 "Daltons".

7. A protein having antiviral interferon activity as claimed in claim 2 appearing at 17,380 ± 100 "Daltons".

8. A protein having antiviral interferon activity as claimed in claim 2 appearing at 17,580 ± 100 "Daltons".

9. A protein having antiviral interferon activity as claimed in claim 2 appearing at 18,410 ± 100 "Daltons".

10. A protein having antiviral interferon activity as claimed in claim 2 appearing at 18,840 ± 100 "Daltons".

11. A protein having antiviral interferon activity as claimed in claim 2 appearing at 19,050 ± 100 "Daltons".

12. A protein having antiviral interferon activity as claimed in claim 2 appearing at 19,500 ± 100 "Daltons".

13. A protein having antiviral interferon activity as claimed in claim 2 appearing at 20,420 ± 100 "Daltons".

14. A protein having antiviral interferon activity as claimed in claim 2 appearing at 20,890 ± 100 "Daltons".

15. A protein having antiviral interferon activity as claimed in claim 2 appearing at 21,380 ± 100 "Daltons".

16. A protein having antiviral interferon activity as claimed in claim 2 appearing at 21,880 ± 100 "Daltons".

17. A protein having antiviral interferon activity as claimed in claim 2 appearing at 22,910 ± 100 "Daltons".

18. An interferon protein having distinct activity in the bovine system, and negligible activity in the human system, the protein appearing at 16,600 ±100 "Daltons".

19. Proteins as claimed in claim 1 having a specific activity of at least $10^8$-$10^9$ IFU per mg protein, as assessed by comparative SDS PAGE staining.

20. Proteins as claimed in claim 19 having a specific activity of $10^8$-$2x10^9$ IFU per mg protein, as assessed by comparative SDS PAGE staining.

21. Protein(s) as claimed in any of the preceding claims, additionally characterized in that it (they) exhibit anticellular activity and potentiate the Natural Killer cell system.

22. A protein as claimed in any of claims 6 - 17, additionally characterized in that it will neutralize antibodies raised against a protein as claimed in any of claims 6 - 17 or raised against proteins as claimed in claim 1, except the 16,600 "Daltons" species.

23. Human leukocyte interferon protein(s) as claimed in any preceding claim.

24. A formulation comprising a protein or proteins as claimed in any of the preceding claims, said formulation being adapted for administration to human beings or animals for prophylactic, therapeutic, or immunization effect.

25. A formulation as claimed in claim 24 adapted for parenteral, intranasal, or topical administration, with or without an added chemostatically active drug.

26. A formulation as claimed in claim 25 which is a cream or ointment for topical administration.

27. A formulation as claimed in claim 24 in the form of a stabilized aqueous solution.

28. A formulation as claimed in claim 27 in which the stabilizer is a protein or a combination of proteins which is non-toxic and non-immunogenic in human beings.

29. A formulation as claimed in claim 28 in which the stabilizer is selected from the group consisting of human serum proteins and

fractions thereof, e.g. and human albumin, or other protein which is non-immunogenic in man.

30. A formulation as claimed in any of claims 24 - 29 in which the concentration of the pure interferon is in the range corresponding to 1 - 40 million IFU per ml.

31. A formulation as claimed in claim 27 for immunizing animals, in which the stabilizer is SDS.

32. A formulation as claimed in claim 31 which is buffered with PBS with a pH of about 7.2.

33. A formulation as claimed in claim 31 or 32 which additionally contains an adjuvant.

34. A formulation as claimed in claim 33 in which the adjuvant is Freund's adjuvant.

35. An SDS complex of a protein or proteins as claimed in any of claims 1 to 23.

36. An SDS complex as claimed in claim 35 isolated in solid form.

37. Antibodies raised against, or directed substantially only against, immunological determinants of human Le form interferon proteins claimed in any of claims 1 - 23.

38. Antibodies as claimed in claim 37 obtained by immunizing an immunizable animal with any of the

16,600, 16,980, 17,380, 17,580, 18,410, 18,840, 19,050, 19,500, 20,420, 20,890, 21,380, 21,880, 22,910

"Daltons" HuIFN-α components of the proteins claimed in claim 1, or with any combination thereof, or obtained from the same components by hybridoma technique or transformation technique.

39. Antibodies as claimed in claim 37 obtained by immunizing an immunizable animal with any of the

16,980, 17,380, 17,580, 18,410, 18,840, 19,050, 19,500, 20,420, 20,890, 21,380, 21,880, 22,910

"Daltons" HuIFN-α components of the proteins claimed in claim 1, or with any combination thereof.

40. Antibodies as claimed in claim 37 obtained by immunizing an immunizable animal with the 16,600 ± 100 "Daltons" HuIFN-α component.

41. Antibodies as claimed in any of claims 37 to 40 obtained by immunizing an immunizable animal with the HuIFN-α component or components obtained from the respective band or bands cut from the SDS PAGE gel.

42. Antibodies as claimed in claim 41 wherein the bands were cut from the SDS PAGE gel after staining of said gel and a short wash in distilled water.

43. A method for preparing antibodies as claimed in claim 37, comprising immunizing an immunizable animal against human Le form protein or proteins as claimed in any of claims 2 - 23 and obtaining antiserum from the animal.

44. A method as claimed in claim 43 in which the immunization is performed with a protein or proteins as claimed in any of claims 2 - 23.

45. A method as claimed in claim 44 in which the immunization is performed with any of the

16,600, 16,980, 17,380, 17,580, 18,410, 18,840, 19,050, 19,500, 20,420, 20,890, 21,380, 21,880, 22,910

"Daltons" HuIFN-α components of the proteins claimed in claim 1, or with any combination thereof.

46. A method as claimed in claim 45 in which the immunization is performed with any of the

16,980, 17,380, 17,580, 18,410, 18,840, 19,050, 19,500, 20,420, 20,890, 21,380, 21,880, 22,910

"Daltons" HuIFN-α components of the proteins claimed in claim 1, or with any combination thereof.

47. A method as claimed in claim 45 in which the immunization is performed with the 16,600 ± 100 "Daltons" HuIFN-α component.

48. A method as claimed in claim 46 in which the immunization is performed with the 21,880 ± 100 "Daltons" component.

49. A method as claimed in any of claims 43 - 48 in which the immunization is performed with a formulation as claimed in any of claims 27 and 31 - 34.

50. A method for producing antibodies as claimed in claim 37, comprising culturing a hybridoma cell clone producing antibodies directed against immunological determinants of HuIFN-α components as claimed in claim 1 and recovering antibodies from the culturing medium.

51. Antibodies when prepared by a method claimed in any of claims 43 - 50.

52. Antibodies as claimed in any of claims 37 - 42 or 51 (or fragments or derivatives thereof retaining the essential anti-interferon determinants) immobilized on a matrix.

53. Antibodies as claimed in claim 52 covalently bound to the matrix.

54. Antibodies as claimed in claim 53 in which the matrix is a cross-linked agarose such as Sepharose 4B.

55. Matrix-immobilized antibodies as claimed in any of claims 51 - 54 which are substantially free from proteolytic enzymatic activity.

56. Matrix-immobilized antibodies as claimed in claim 55 which have been substantially freed from any proteolytic enzymatic activity by treatment with enzyme inhibitors or enzyme destructors.

57. Matrix-immobilized antibodies as claimed in claim 57 for which the treatment with enzyme inhibitors or enzyme destructors has been carried out with matrix-immobilized enzyme inhibitor or enzyme destructor.

58. Matrix-immobilized antibodies as claimed in claim 57 which, prior to their covalent binding to the matrix, have been passed through a column of matrix-immobilized poly-L-lysin and/or matrix-immobilized Soyabean Trypsin inhibitor, and/or matrix-immobilized kallikrein inactivator.

59. The use of antibodies as claimed in any of claims 37 - 42 and 51 - 58 for purifying human Le form interferon-containing solutions.

60. A protein having the significant interferon-characterizing determinants possessed by the proteins claimed in any of claims 1 - 23.

61. A protein having the significant immunological determinants possessed by the proteins claimed in any of claims 1 - 23.

62. A method of producing human Le form interferon proteins as claimed in claim 1 or 3 - 5, comprising subjecting a solution containing human Le form interferon proteins to affinity chromatography such that said human Le form interferon proteins are selectively retained, and eluting said retained interferon proteins.

63. A method as claimed in claim 42, wherein the affinity chromatography comprises antibody affinity chromatography in which the antibodies are matrix-immobilized antibodies as claimed in any of claims 52 - 58.

64. A method as claimed in claim 63 in which at most 85% of the antibodies which were applied to the matrix at the immobilization stage became covalently bound.

65. A method as claimed in claim 63 or 64 in which the antibodies, prior to being bound to the matrix, had been substantially freed from any proteolytic enzymatic activity by treatment with enzyme inhibitors and/or enzyme destructors.

66. A method as claimed in claim 65 in which the removal of the proteolytic activity had been carried out with matrix-immobilized enzyme inhibitor and/or enzyme destructor.

67. A method as claimed in any of claims 62 - 66 in which the solution applied to the antibody affinity matrix is a crude, unconcentrated interferon preparation.

68. A method as claimed in any of claims 62 - 66 in which the solution applied to the antibody affinity matrix is a concentrated or partially purified interferon preparation.

69. A method as claimed in any of claims 62 to 66 in which the interferon-containing solution comprises the interferon-containing

protein fraction obtained by a protein precipitation treatment of a crude, unconcentrated interferon preparation.

70. A method as claimed in 69 in which the protein precipitation treatment includes the addition of KSCN and the adjustment of the solution pH to 4.5.

71. A method as claimed in any of claims 62 - 70 in which the solution applied to the antibody affinity matrix is an interferon solution which essentially only contains proteins in the 10,000 - 20,000 Daltons range.

72. A method as claimed in claim 71 in which the solution is the 10,000 - 20,000 Daltons eluate from a gel filtration performed with a buffer solution which contains about 25% by volume of a glycol such as ethylene glycol and has an ionic strength corresponding to 1 M NaCl, pH about 7.2.

73. A method as claimed in any of claims 63 - 72 in which the HuIFN-α interferon of the solution applied to the antibody affinity matrix is selected from the group consisting of human leukocyte interferons, human lymphoblastoid interferons, and proteins as claimed in claim 60 or 61, including such proteins when produced by cultivation of a microorganism carrying DNA coding for the production of such proteins.

74. A method as claimed in claims 73 in which the interferon-containing solution comprises the interferon-containing protein fraction obtained by a protein precipitation treatment of a crude, unconcentrated interferon preparation.

75. A method as claimed in 74 in which the protein precipitation treatment includes the addition of KSCN and the adjustment of the solution pH to 4.5.

76. A method as claimed in any of claims 73 - 75 in which the solution applied to the antibody affinity matrix is an interferon

solution which essentially only contains proteins in the 10,000 - 20,000 Daltons range.

77. A method as claimed in claim 76 in which the solution is the 10,000 - 20,000 Daltons eluate from a gel filtration performed with a buffer solution which contains about 25% by volume of a glycol such as ethylene glycol and has an ionic strength corresponding to 1 M NaCl, pH about 7.2.

78. A method as claimed in any of claim 74 to 77 wherein the eluate in the gel filtration buffer is loaded directly on the antibody affinity matrix.

79. HuIFN-α interferon when prepared by the use of matrix-immobilized antibodies as claimed in any of claims 52 - 58 or by a method as claimed in any of claims 62 - 78.

80. HuIFN-α interferon as claimed in claim 79, having a specific activity of at least $30x10^6$ IFU per mg protein, the protein determination being based on the Lowry procedure using pure human albumin serum as standard.

81. HuIFN-α interferon as claimed in claim 80 having a specific activity in the range from $30x10^6$ to $10^8$ IFU per mg protein

82. HuIFN-α interferon as claimed in claim 81 having a specific activity of $30x10^6$ - $70x10^6$ IFU per mg protein.

83. A method of producing interferon proteins or proteins having the significant biological interferon-activity determinants thereof, comprising cultivating a microorganism carrying DNA coding for the production of HuIFN-α interferon proteins as claimed in any of claims 1 - 23 or 60, and recovering said proteins from the culture medium.

84. A method as claimed in claim 83 in which the DNA coding for the production of interferon proteins is present on a plasmid.

85. A method as claimed in claim 83 or 84 in which the DNA coding for the production of the interferon proteins has been prepared by treatment, with reverse transcriptase, of messenger RNA isolated from interferon-producing cells in a manner known per se and comprising the stage of antibody affinity chromatography and/or immunoprecipitation on a lysate of the cells, the antibody being used in the antibody affinity chromatography or in the immunoprecipitation being an antibody as claimed in any of claims 37 - 42 or 51.

86. A method as claimed in any of claims 83 - 85 in which the interferon-producing clones of the microorganism have been selected by means of radio-labelled monospecific antibodies as claimed in any of claims 37 to 42 or 51.

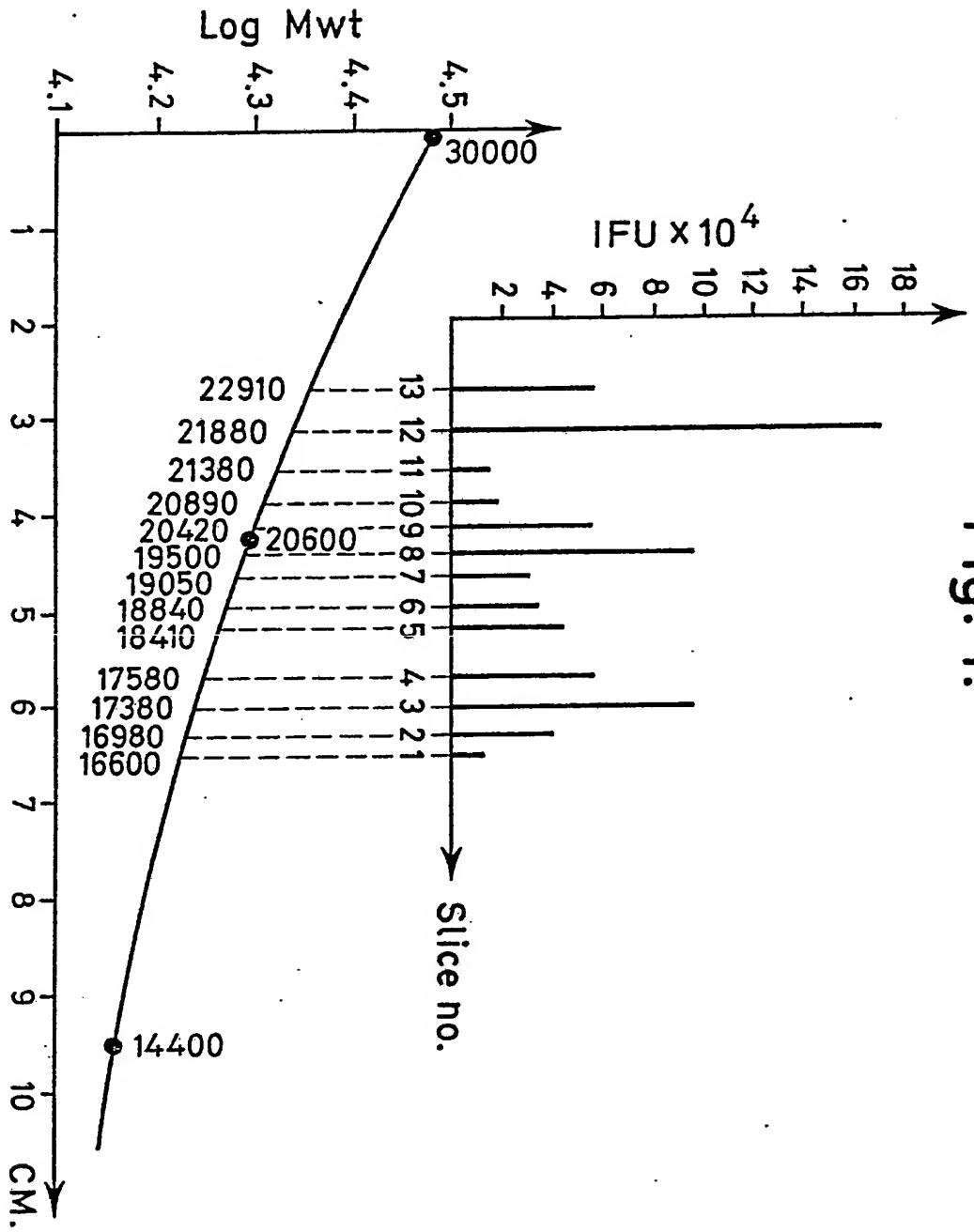


Fig. 1.

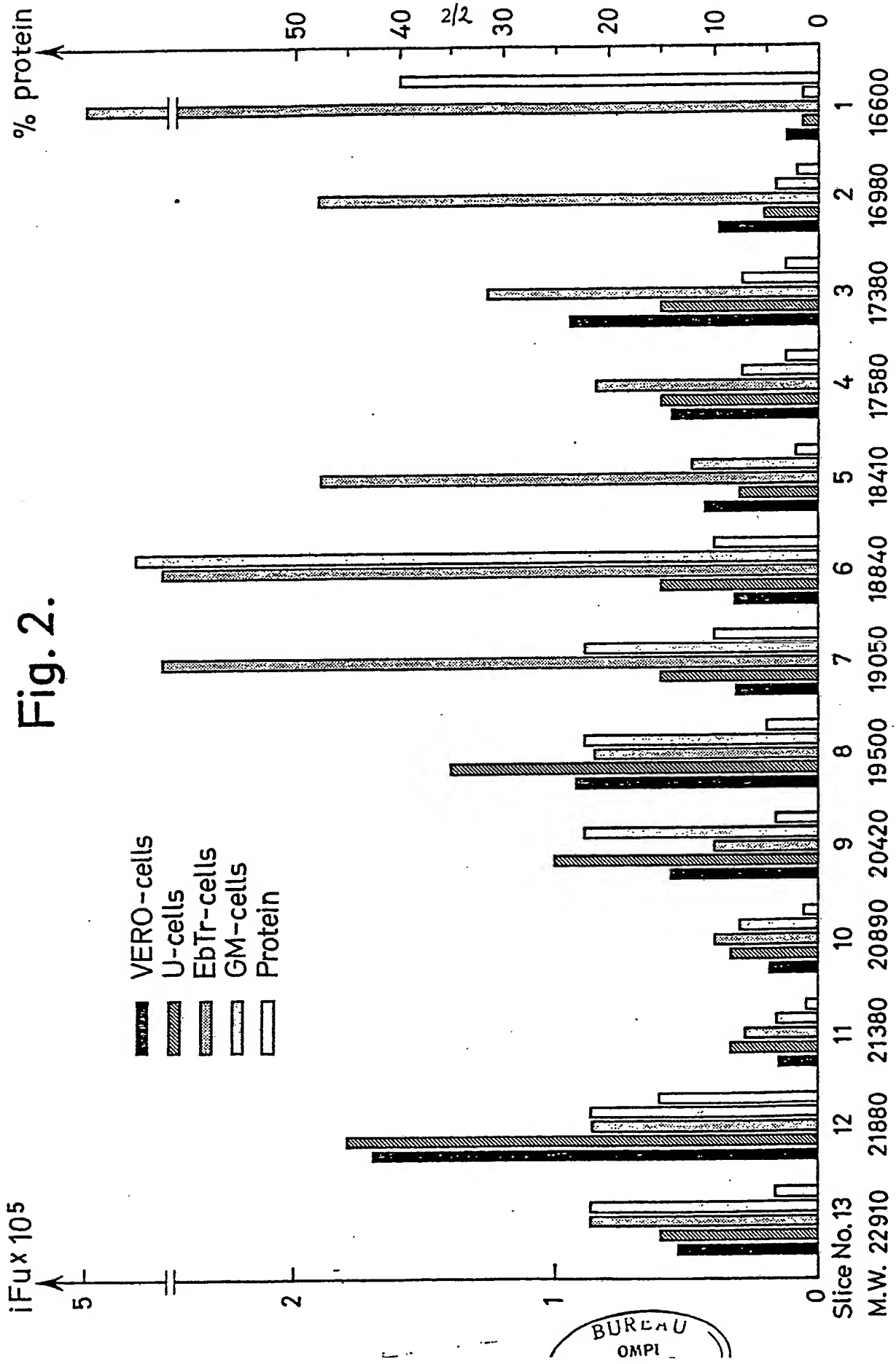


Fig. 2.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK82/00077

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 07 G 7/00, A 61 K 45/02 // C 12 N 5/00, 15/00, A 61 K 39/395

**II. FIELDS SEARCHED**

**Minimum Documentation Searched** [4]

| Classification System | Classification Symbols |
|---|---|
| IPC 3 | C 07 G 7/00, C 12 N 15/00, C 12 P 21/00, 21/02<br>A 61 K 45/02 |
| US Cl | 424:85; 435:68; 260:112 |

**Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched** [5]

SE, NO, DK, FI classes as above

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| X | Chemical Abstracts Vol 95 (1981), abstract No 78332r, Arch. Biochem. Biophys. 1981, 210(1), 307-18. | 1-23 |
| X | Chemical Abstracts Vol 93 (1980), abstract No 24156b, Scand. J. Immunol. 1980, 11(15), 489-502. | 1-5,9,12,14 19-23,35-46 |
| X | Ann. New York Acad. Sci. Vol 350, pp 594-5, published October 1980 (BERG KURT et al) "The complete purification of human leukocyte interferon". | 1-5,9,12,14 19-23,35-46 |
| X | Proc. Natl. Acad. Sci. USA Vol 76, No 11, pp 5601-05, published November 1979 (ZOON K C et al), "Purification and partial characterization of human lymphoblastoid interferon". | 9,37,43-6, 49-53,59-64 67-82 |
| A | J. gen. Virol. Vol 39, pp 125-30, published 1978 (LIN L S et al), "Characterization of the heterogeneous molecules of human interferons: Differences in .../... | |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 1982-11-10 | 1982 -11- 15 |
| **International Searching Authority** [1] | **Signature of Authorized Officer** [20] |
| Swedish Patent Office | Carl Olof Gustafsson |

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .. ... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... ..., because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this International application as follows:

See enclosure 1.

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention ~~first mentioned in the claims; it is~~ covered by claim numbers:

1-42, 51-59 and 62-82.

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, 16 with indication, where appropriate, of the relevant passages 17 | Relevant to Claim No 18 |
|---|---|---|
| | .../... the cross-species antiviral activities of various molecular populations in human leukocyte interferons". | |
| A,X | J. Biol. Chem. Vol 253, No 14, pp 5206-12, published 1978, (BERTHOLD W et al), "Purification and in vitro labeling of interferon from a human fibroblastoid cell line". | 1, 11 |
| Y | Science, Vol 209, pp 1343-47, published 19 September 1980 (STREULI M et al), "At least three human type α interferons: structure of α2". | 1-23 |
| A | J. Biol. Chem. Vol 256, pp 3770-75, published 25 April 1918 (YONEHARA S et al), "Purification of human lymphoblastoid interferon by a simple procedure with high yields". | |
| X | WO 80/02229 (A/S ALFRED BENZON) 30 October 1980 & EP, A, 18 218 | 1,2,9,12, 13,19-39, 41-46,49-86 |
| X | GB, A, 2 037 296 (F HOFFMANN-LA ROCHE & Co AG) 9 July 1980 | 1-23 |
| P | WO, A, 81/02899 (SECHER D S,BURKE D) 15 October 1981 | 37-59, 62-82 |
| | EP, A, 32 134 (BIOGEN N V) 15 July 1981 | |
| | EP, A, 43 980 (GENENTECH INC) 20 January 1982 | |
| | EP, A, 51 873 (GENENTECH INC) 19 May 1982 | |
| Y,X | Nature Vol 290, pp 20-26, published 5 March 1981 (GOEDDEL D V et al), "The structure of eight distinct cloned human leukocyte interferon cDNAs". | 1-23, 83-86 |
| Y/P | Chemical Abstracts 96 (1980), abstract No 63617j, Gene 1981, 15(4), 379-94. | 1-23, 83-86 |

Enclosure 1

Inventions:

| | |
|---|---|
| A. Claims 1-23 (60, 61, 79-82) | Interferon. |
| Claims 62-78 | Method for the production/ purification of Interferon. |
| B. Claims 24-34 | Pharmaceutical formulation. |
| C. Claims 37-42, 51-58 | Antibodies to interferon. |
| Claims 43-50 | Method for the production of antibodies to interferon. |
| Claim 59 | Use of the antibodies for the purification of interferon. |
| D. Claims 83-86 | Method for the production of interferon. |

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